Seahorse brood pouch morphology and control of male parturition in *Hippocampus abdominalis*

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Abstract

Introduction: Syngnathids (seahorses, pipefishes and seadragons) are among the few vertebrates that display male pregnancy. During seahorse pregnancy, males incubate developing embryos embedded in a placenta within a fleshy brood pouch, before expelling fully developed neonates at parturition. The mechanisms underpinning seahorse parturition are poorly understood.

Methods: We examined the morphology of the brood pouch using microcomputed tomography and histological techniques, in combination with physiological assays, to examine how male pot-bellied seahorses (*Hippocampus abdominalis*) control labour. In female-pregnant vertebrates, nonapeptide hormones (such as vasopressin- and oxytocin-like hormones) produce contractions of gestational smooth muscle to produce labour.

Results: Histological analysis of the seahorse brood pouch reveals only scattered small smooth muscle bundles in the brood pouch, and *in-vitro* application of isotocin (a teleost nonapeptide hormone) to the brood pouch do not produce measurable muscle contractions. Micro-computed tomography shows differences in size and orientation of the anal fin assembly between male and female pot-bellied seahorses, and histological analysis reveals large skeletal muscle bundles attached to the anal fin bones at the male brood pouch opening.

Discussion: We conclude that seahorse parturition may be facilitated by contraction of these muscles, which, in combination with body movements, serves to gape open the pouch and expel the neonates. Future biomechanical studies are needed to test this hypothesis.

Key words

Anal fin musculature, brood pouch, contraction assay, labour, μ-CT, syngnathid, pregnancy

Highlights

- Male seahorses incubate embryos inside a brood pouch via a placenta
- We examined the mechanisms by which parturition is controlled in male seahorses
- *In vitro* application of isotocin did not produce measurable brood pouch contractions
- A modified anal fin assembly in males may act in combination with body movements to expel neonates
- Provides evidence for divergent mechanisms underpinning parturition in pregnant vertebrates

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21 ## Abstract

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23 vertebrates that display male pregnancy. During seahorse pregnancy, males incubate
24 developing embryos embedded in a placenta within a fleshy brood pouch, before expelling
25 fully developed neonates at parturition. The mechanisms underpinning seahorse parturition
26 are poorly understood.
27 Methods: We examined the morphology of the brood pouch using microcomputed
28 tomography and histological techniques, in combination with physiological assays, to
29 examine how male pot-bellied seahorses (*Hippocampus abdominalis*) control labour. In
30 female-pregnant vertebrates, nonapeptide hormones (such as vasopressin- and oxytocin-like
31 hormones) produce contractions of gestational smooth muscle to produce labour.
32 Results: Histological analysis of the seahorse brood pouch reveals only scattered small
33 smooth muscle bundles in the brood pouch, and *in-vitro* application of isotocin (a teleost
34 nonapeptide hormone) to the brood pouch do not produce measurable muscle contractions.
35 Micro-computed tomography shows differences in size and orientation of the anal fin
36 assembly between male and female pot-bellied seahorses, and histological analysis reveals
37 large skeletal muscle bundles attached to the anal fin bones at the male brood pouch opening.
38 Discussion: We conclude that seahorse parturition may be facilitated by contraction of these
39 muscles, which, in combination with body movements, serves to gape open the pouch and
40 expel the neonates. Future biomechanical studies are needed to test this hypothesis.
41

42 **Key words**
43 Anal fin musculature, brood pouch, contraction assay, labour, μ-CT, syngnathid, pregnancy
44
45

46 ## Introduction

47

48 All species of Syngnathidae (seahorses, pipefishes and seadragons) exhibit male pregnancy
49 [reviewed in 1, 2, 3]. Developing embryos are incubated on or in a brood pouch structure
50 produced by outgrowths of tail or abdominal epithelium [4], the complexity of which varies
51 between species [reviewed in 2, 3, 5]. Seahorses have the most complex brood pouch,
52 incubating embryos via placentae sealed within a thick, fleshy structure [reviewed in 2, 3, 5].
53 Seahorse brood pouches provide a range of physiological support to embryos, including
54 respiratory gas and waste exchange, osmoregulation, nutrient supplementation, and
55 immunological protection [e.g. 2, 3, 6, 7-13].

56

57 The endocrine control of syngnathid pregnancy is poorly understood compared to model
58 teleosts [see review by 14]. Syngnathid brood pouch development and the progression of
59 pregnancy are at least partially controlled by androgens [14-16]. Kisspeptin and
60 gonadotropin-inhibitory hormone may play a role in regulating gonadotropin-releasing
61 hormone and testosterone during puberty and pregnancy (Zhang *et al.* 2018, Zhang *et al.*
62 2019), with prolactin and adrenocorticotrophic hormone helping to maintain pregnancy [14,
63 15, 17]. 17α-hydroxy-20β-dihydroprogesterone may also be involved [18]. While the
64 endocrinology of syngnathid parturition is poorly understood, nonapeptide hormones
65 (vasopressin-like and oxytocin-like hormones) are likely involved. Brain concentrations of
66 arginine vasotocin are elevated during pregnancy in male pipefishes *Syngnathus* spp. [19].
67 Administration of an endocrine disruptor (Aroclor 1254) to these pipefishes increases brain
68 arginine vasotocin concentrations [20] and causes earlier parturition [21]. In seahorses,
69 injection of oxytocin and isotocin (the teleost ortholog of oxytocin) into the pouch wall of
70 non-pregnant males induces parturition-like behaviours [22].

71

72 In female-pregnant vertebrates (viviparous mammals, reptiles, and fishes), nonapeptide
73 hormones are major mediators of parturition through their action of inducing contractions in
74 the smooth muscles of the gestational tissues (uterus and ovary) [23-27]. *In vitro* assays of
75 gestational tissues in tetrapods produces contraction of gestational smooth muscle in response
76 to nonapeptide administration [e.g. 27, 28, 29-32]. These hormones also cause contractions of
77 ovarian and oviductal smooth muscle and elicit parturition in viviparous teleost fishes [e.g.
78 27, 33, 34] and are implicated in producing uterine contractions in elasmobranchs [27]. Given

79 the presence of smooth muscle in the seahorse brood pouch [4], the effects of exogenous
80 isotocin on non-pregnant males [22], and the similarities in genes underpinning parturition in
81 male seahorses and pregnant female amniotes [12], we predicted that nonapeptide hormones
82 play a significant role in producing labour in seahorses.
83
84 Here we aimed to test the hypothesis that isotocin provides the physiological trigger for
85 parturition in male seahorses by determining the *in vitro* effect of isotocin on brood pouch
86 tissue. We also test the hypothesis that specific anatomical structures facilitate parturition in
87 male seahorses by characterising the bone and muscle structures of the brood pouch opening.

88 Methods

89 *Animal husbandry*
90 *Hippocampus abdominalis* seahorses (captive-bred by Seahorse Australia, Tasmania) were
91 housed together in 75 L aquaria at 18 ℃, and fed enriched frozen *Mysis relicta* as previously
92 described [10] under University of Sydney Animal Ethics Committee approval number 2018-
93 1302. Tank temperature, salinity, pH, nitrate, nitrite, and ammonia were monitored to ensure
94 optimal water quality. Sexually mature, non-brooding seahorses (three pregnant males for the
95 contraction assays; two males and two females for μ-CT; two males and two females for
96 histology) were humanely euthanised under University of Sydney Animal Ethics Committee
97 approval number 2018-1302 and University of Newcastle Animal Care and Ethics Committee
98 permit number A2016-620. Female seahorses were included in the morphological assays to
99 compare the differences in orientation and structure of reproductive tissues and the
100 surrounding anatomical structures.
101
102 *Brood pouch contraction assays*
103 Seahorse brood pouch tissue from three late pregnant males (at least at the protruding snout
104 stage >70 % of the way through development [35]) was cut into strips (~10 × 2 × 2 mm) and
105 mounted on MLT0201/RAD force transducers (ADInstruments, Bella Vista, NSW, Australia)
106 using nylon thread, as previously described [36, 37]. We assessed each pouch using strips
107 dissected in three different orientations: vertically, horizontally or diagonally, to account for
108 potential variation in muscle fibre direction [38] in the brood pouch. For each animal, two
109 strips were cut in each direction; one of each pair was assessed in Munsick's solution and the
110 other was assessed in marine Ringer's solution. For each animal, strips of seahorse
111 gastrointestinal tract were also included as positive controls for each buffer, tied

112 longitudinally as a whole tube. Organ bath transducers were calibrated to 2 g at 1 mV. Each
113 strip of tissue was lowered into one of eight drainable organ baths (Radnotti 159920)
114 containing 17 mL of either Munsick's solution (113 mM NaCl, 6 mM KCl, 0.5 mM $CaC1_2$, 1
115 mM NaH_2PO_4, 30 mM $NaHCO_3$, 1.6 mM glucose) or marine Ringer's solution (11294 mM
116 NaCl, 496 mM KCl, 218 mM $CaCl_2$:$2H_2O$, 101 mM $MgSO_4$:$7H_2O$, 101 mM $NaHCO_3$, 499
117 mM Hepes buffer, 499 mM Glucose, 101 mM Na_2HPO_4:$7H_2O$) [39]. All organ baths were
118 continuously gassed with carbogen (95% O_2, 5% CO_2) and organ bath temperature was
119 maintained at 18 °C (the temperature of the aquaria in which the animals are housed) via a
120 circulating water bath. Passive tension of 1.0 g was applied to each brood pouch strip and 0.5
121 g to the gut strip by adjusting the transducer positions. Such stretching induces spontaneous
122 contraction of smooth muscle [reviewed in 40]. Organ bath buffer was replaced three times at
123 ten-minute intervals, with tension reset to either 1.0 g for brood pouch and 0.5 g for gut each
124 time. After re-tensioning, the tissue was incubated for a further 1 h to allow any spontaneous
125 contractility [41] to stabilise.
126
127 *Dose response*
128 Synthetic isotocin (amino acid sequence CYISNCPIG; FW: 966.1; Cat# 309165, NovoPro
129 Biosciences) dissolved in 15 % acetonitrile (ACN) (isotocin stock concentration of 1.31
130 mM), was used in this assay as this hormone produces ovarian contractions in a teleost fish
131 [42]. A dose response pilot study was conducted on gastrointestinal tract and brood pouch
132 tissue of two animals (one non-pregnant, one with early embryos), but as no brood pouch
133 contractile response was elicited even at the highest concentration of 10 µM, 10 µM isotocin
134 was used for treatments for the three late-pregnant experimental animals thereafter. 130 µL of
135 the peptide buffer (15 % ACN in H_2O) was added first to each bath as a vehicle control. After
136 10 min, 130 µL of isotocin was pipetted into the organ baths (10 µM final concentration) and
137 contractile responses were recorded for 10 min. Then, two rounds of potassium chloride
138 (KCl) were administered to each organ bath to serve as positive controls, as KCl directly
139 depolarises the membrane to induce contractile activity in uterine (and other) smooth muscle
140 [43]. First, 1 M KCl was added up to a final concentration of 10 mM and contractile
141 responses recorded for 10 min. Afterwards, further 1 M KCl was added to each organ bath up
142 to a final concentration of 40 mM, and contractile responses recorded for a further 10 min.
143
144 *µ-CT scans*

Entire animals were fixed in 10 % neutral buffered formalin (NBF) for two weeks. Specimens were then immersed in a solution of 0.3 % Phosphotungstic acid (PTA) in 70% ethanol for four weeks, rotating every few days and changing solution every two weeks. PTA binds to collagen and other proteins and musculature is demonstrated distinctly in tomographic images; cartilage does not stain strongly with PTA, but appears as gaps in volume renderings ([44]). After staining, specimens were rinsed in 100% ethanol to remove unbound stain, wrapped in parafilm and mounted in plastic tubing. Zeiss Xradia MicroXCT-400 with the source set to 50 kV and 200µA. Multiple scans were performed to capture the entirety of each sample. Consistent reconstruction parameters were applied to the individual scans allowing for 3D registration and stitching to create image stacks which were then volume rendered using Avizo (Thermo Fisher Scientific).

Histology

Decalcification, Fixation & Embedding

Entire animals were fixed in 10 % NBF for two weeks. The samples were decalcified in changes of 70% EDTA every 4 - 7 days over two months then stored in 70% ethanol (changed once a week) until embedding. Animals were divided into several sections to fit into embedding cassettes: head, abdomen (top/mid/bottom, or top/mid/bottom pouch if male), tail and tail end. Cassettes containing animal samples were placed into a tissue processor (Thermo Scientific Excelsior ES, Thermo Scientific US) for approximately 13 h to allow infiltration of paraffin wax through all cavities. The samples were then embedded into paraffin wax blocks in embedding cassettes along sagittal and transverse aspects using an embedding machine (Tissue-Tek TEC 5, Sakura US).

Sectioning & Staining

Serial sections (transverse and sagittal) throughout the entire brood pouch (male) or abdomen (female) were taken at 7 µm thickness with a microtome (Thermo Scientific Microm HM 325, Thermo Scientific US). Every fifth slide (approx. every 35 ± 7 µm) for transverse sections and every third slide (approx. every 21 ± 7 µm) for sagittal sections were stained with modified Cason's Trichrome [45] to discriminate between muscle (red staining) and connective tissue (blue staining). The staining process included clearing with histolene (5 min, two changes), hydration through an ethanol series (100% > 100% > 95% > 70%, 3 min each), immersion in Bouin's fixative (75 ml saturated picric acid, 25 ml 100% NBF, 5 ml glacial acetic acid) for 2 h at 60°C, and rinsing well with distilled water before staining with

179 Cason's Trichrome (Phosphotungstic acid crystals 1 g, Orange G 2 g, Aniline blue 1 g and
180 Acid fuchsin 3 g in 200 mL distilled water) for 4 min [46]. The sections were then
181 dehydrated in a reverse ethanol series (70% > 95% > 100% > 100%, 1 min each) before
182 clearing in histolene (3 min, two changes) and mounting in Dibutylphthalate Polystyrene
183 Xylene. Digital images of slides were scanned using an Axio Scan.Z1 (ZEISS, Germany)
184 microscope.
185

186 **Results**

187 *Brood pouch contraction bioassays*

188 Gastrointestinal tract (positive control tissue) from all 3 late-pregnant males contracted in
189 response to treatment with KCl (10 and 40 μM) when suspended in either Munsick's or
190 marine Ringer's solution, indicating that both solutions were appropriate buffers.
191 Nonetheless, we noted that gastrointestinal tract from 2 out of 3 pregnant males exhibited
192 spontaneous contractility (representative trace in Figure 1) when suspended in Munsick's
193 solution, whereas spontaneous contractility was not observed when gastrointestinal tract was
194 suspended in marine Ringer's solution (representative trace in Figure S1), which may
195 indicate that Munsick's was the more appropriate buffer. Gastrointestinal tract from all three
196 pregnant males contracted in response to treatment with isotocin (10 μM) in both Munsick's
197 and Ringer's solution, indicating that the isotocin preparation was bioactive, whereas
198 matched volumes of peptide buffer (15% ACN) elicited no contractile response.
199

200 In contrast to gastrointestinal tract, brood pouch tissue from pregnant male *H. abdominalis*
201 (n=3) produced no measurable contractility in response to isotocin (10 μM), regardless of
202 vertical, horizontal or diagonal dissection orientation, and whether the tissues were suspended
203 in Munsick's (Figure 1) or marine Ringer's solution (Figure S1). Additionally, brood pouch
204 tissue produced no measurable contractility in response to KCl at concentrations that
205 effectively elicited contractile responses from gastrointestinal tract (10 and 40 μM).
206
207

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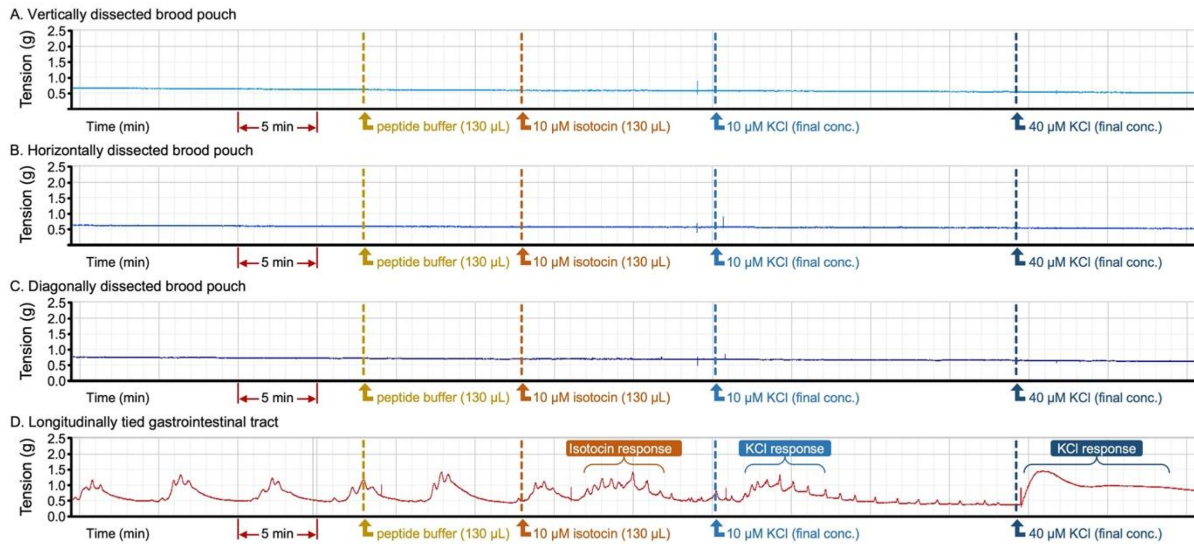


209

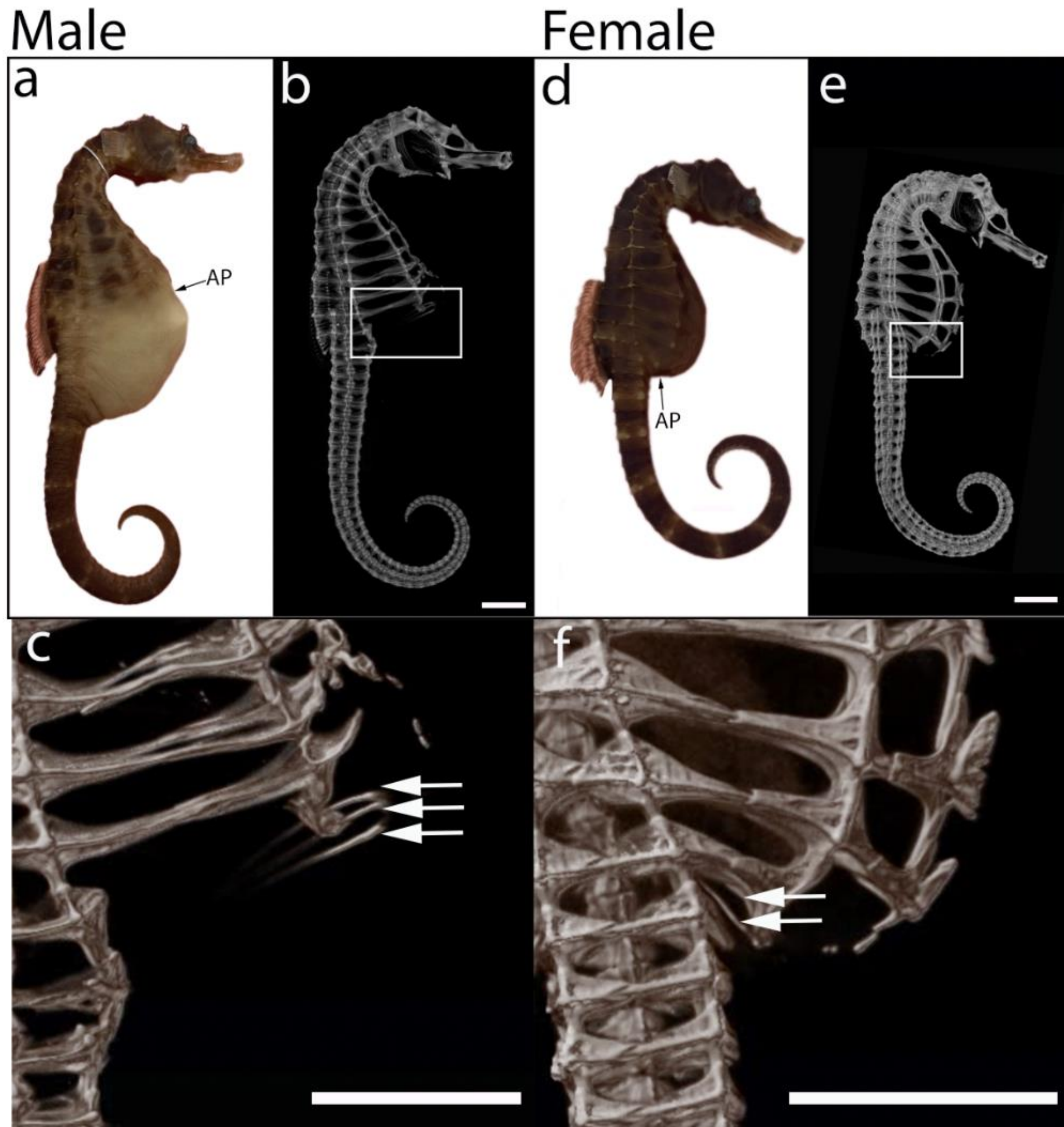
210 ***Figure 1.*** Representative partial contraction traces for individual strips of seahorse brood
211 pouch tissue (panels A - C) and gastrointestinal tract (positive control; panel D) suspended in
212 Munsick's solution. Representative data from a single late-stage pregnant male seahorse.
213 Panels A - C are seahorse brood pouch tissue cut in vertical, horizontal, and diagonal
214 orientation, respectively. Panel D is longitudinally tied gastrointestinal tract. Brood pouch
215 tissue (of any dissection orientation) did not contract in response to isotocin (10 μM) or KCl
216 (10 and 40 μM), whereas gastrointestinal tract contracted in response to both isotocin and
217 KCl. Consistent results were observed when tissues were suspended in marine Ringer's
218 solution (Figure S1).

219

220 *μ-CT scanning*

221 The external and skeletal morphology of *H. abdominalis* is different between the sexes.
222 Males have a fleshy brood pouch caudal to the trunk (Figure 2a), which does not contain
223 bony rings (Figure 2b, c). The bodies of female *H. abdominalis* have bony rings running
224 along the entire ventral aspect of the abdomen and the tail (Figure 2d, e). The orientation of
225 the most caudal trunk ring differs between males and females: it is consistently angled
226 caudally in females (Figure 2f) and cranially in males (Figure 2c). The anal fin assembly, of
227 which the pterygiophores (see Histology section of results) are visible in the scans, is located
228 caudal to the most caudal trunk ring in both sexes (Figure 2c, f). The anal fin pterygiophores
229 are longer in males than females (Figure 2, Video S1 and S2).

230



231

232 ***Figure 2:*** Comparison of photographs, and μ-CT scan renderings of male and female
233 *Hippocampus abdominalis*. *(a)* The external features of male *H. abdominalis* and the location
234 of the anal pore (AP) and fleshy brood pouch caudal to the trunk. *(b)* μ-CT scan renderings
235 showing the skeletal structure of male *H. abdominalis,* highlighting the position of the anal
236 fin assembly (white box). *(c)* large pterygiophores are angled cranially in males (white
237 arrows). (*d)* The external features of female *H. abdominalis* and the location of the anal pore
238 (AP) caudal to the trunk and dermal plates that are positioned along the entire ventral aspect
239 of the abdomen*. (e)* μ-CT scan renderings showing the skeletal structure of female *H.*
240 *abdominalis,* highlighting the position of the anal fin assembly (white box). *(f)* small

241 pterygiophores angled caudally (white arrows). One pterygiophore is obscured by a tail ring
242 in this image. Scale bars indicate 1 cm.
243
244 *Histology*
245 *Comparison between male and female*
246 Large skeletal muscle bundles are present at the base of the tail in both sexes (Figure 3,
247 Figure 4), and are the anal fin musculature previously identified by Consi *et al*. [47]. This
248 musculature is consistently larger in males than females. Seahorses have a similar basic anal
249 fin structure to other teleosts (e.g. zebrafish [48]). The fin assembly consists of bony fin rays
250 supported by fin ray muscles and cartilaginous pterygiophores. The pterygiophores attach to
251 the ventral side of the vertebrae and are made up of (in order from spine to distal end of fin):
252 proximal radial, middle radial, and distal radial; fin ray bones attach to each distal radial [47].
253 The fin ray muscles consist of two pairs of muscles per pterygiophore (a pair of depressors,
254 and a pair of inclinators; Figure 3 b, c), which attach to the base of each fin ray [47].
255
256 *Female seahorse*
257 The paired ovaries filled with lipid-rich oocytes dominate the abdominal cavity (Figure 3a).
258 The anal fin is located caudal to the anal pore (Figure 3a). Skeletal muscles stain red with
259 Cason's Trichrome; both inclinator (*inclinatores anales*, which control sideways motion of
260 the anal fin) and depressor (*depressores anales*, which erect or depress the anal fin) muscles
261 are visible (Figure 3b, c). Due to the plane of sectioning, only the proximal radials of the
262 cartilaginous anal fin pterygiophores are visible (Figure 3b, c). The skeletal musculature of
263 the anal fin is caudal to the abdomen of *H. abdominalis*, extending from the ventral surface of
264 the vertebra to the base of the abdomen. Pairs of *inclinatores anales* and *depressores anales*
265 are visible, which are each attached to a proximal radial cartilage and a middle radial
266 cartilage of the anal fin [47] (Figure 3c, d; attachments not visible due to the plane of
267 sectioning).
268

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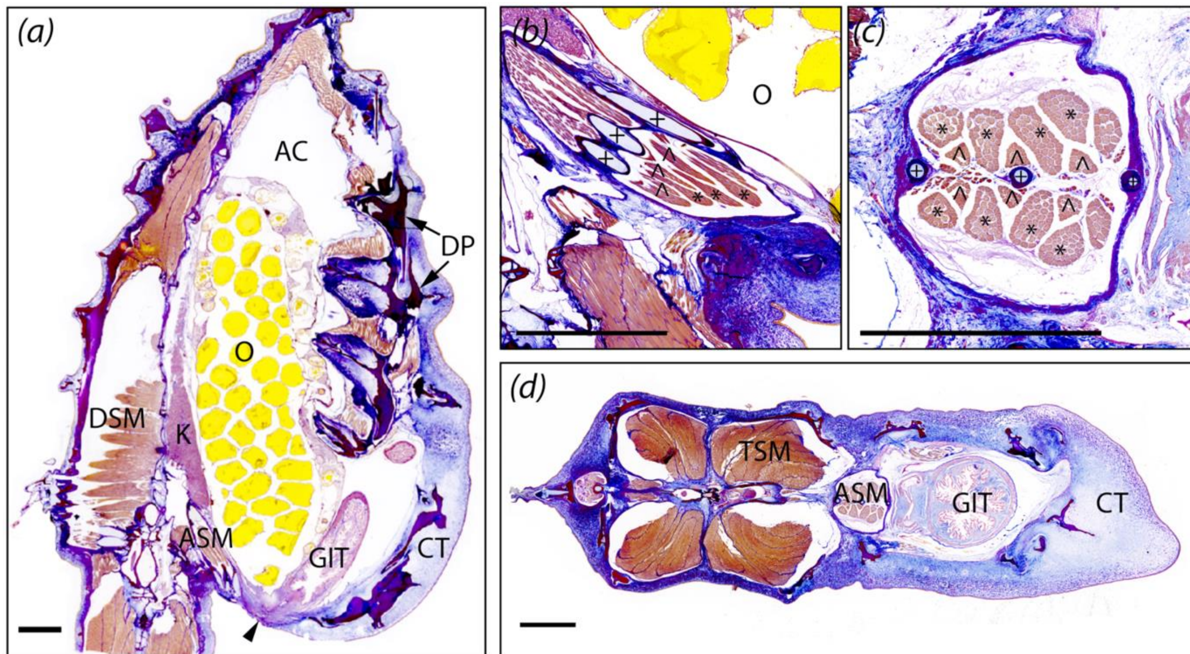
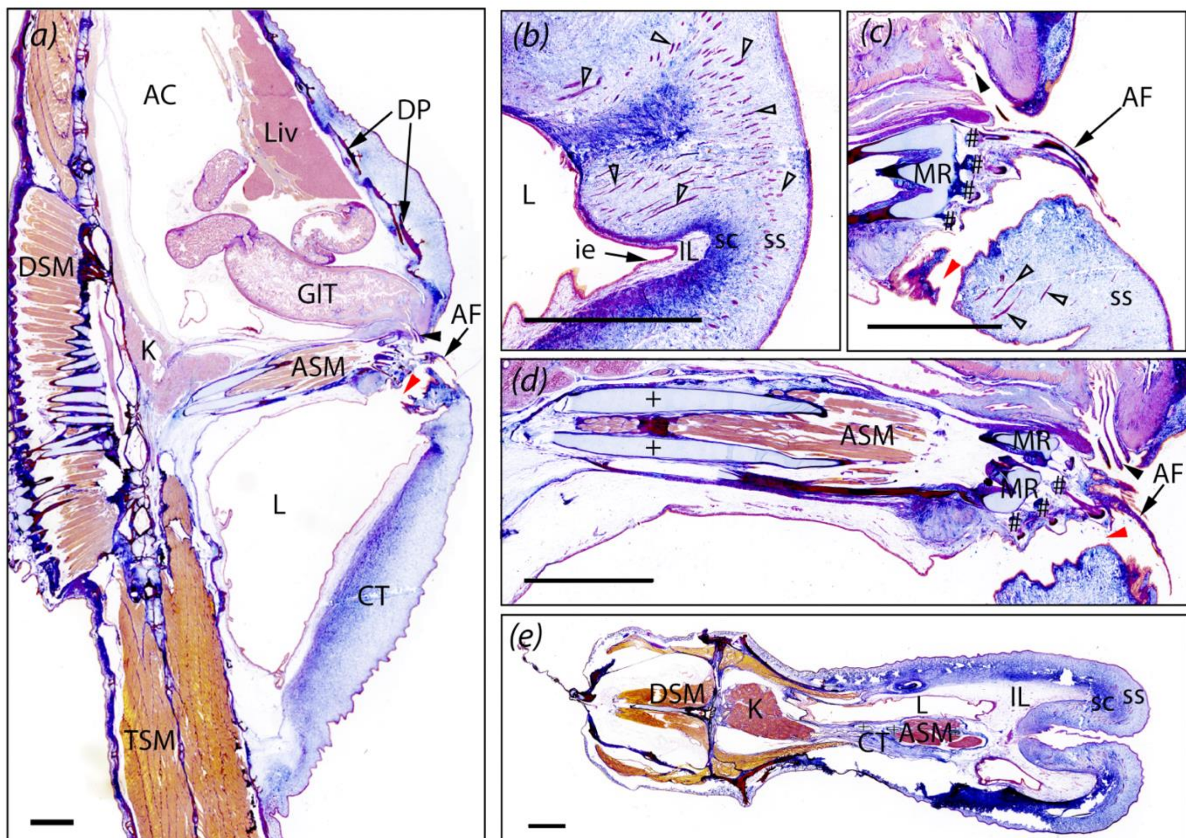


270 ***Figure 3.*** Whole body sections of a female seahorse, *Hippocampus abdominalis*, excluding
271 the head and distal tail, stained with Cason's Trichrome. *(a)* A sagittal section of a female
272 seahorse, off from the midline (hence the anal fin rays are not visible). The ovary (O) of the
273 female seahorse occupies a large portion of the abdominal cavity (AC) and is situated ventral
274 to the kidney (K). The oocytes are stained yellow. The anal fin skeletal muscle assembly
275 (ASM) is small and located between the abdomen and the spine. The anal pore (black
276 arrowhead) is not entirely visible, as this sagittal section is not along the midline of the
277 animal. *(b)* Higher magnification of the anal fin musculature and pterygiophores
278 demonstrates cartilaginous radials (+) of the pterygiophores and *depressores anales* (^), and
279 *inclinatores anales* (*) of the anal fin skeletal muscle. *(c)* High magnification of the anal fin
280 musculature and pterygiophores from a transverse section of a female seahorse. The paired
281 *inclinatores anales* (*) and *depressores anales* (^) are positioned on either side of the radials
282 (+). *(d)* Whole transverse section of a female seahorse at the base of the abdomen, cranial to
283 the anal pore. The tail skeletal muscle (TSM) and anal fin skeletal muscles (ASM) are dorsal
284 to the gastrointestinal tract (GIT). All scale bars represent 2000 µm. Abdominal cavity (AC),
285 anal fin skeletal muscle assembly (ASM), anal fin (AF), connective tissue (CT), dermal
286 plates (DP), dorsal fin skeletal muscle (DSM), gastrointestinal tract (GIT), anal pore (black
287 arrowhead), kidney (K), tail skeletal muscle (TSM), radial (+), ovary (O), *inclinatores anales*
288 (*), *depressores anales* (^).

289

290 *Male seahorse*

291 The fleshy brood pouch of *H. abdominalis* extends ventrally over the base of the tail (Figure
292 4a) and consists of several layers: an inner layer of loose connective tissue; the middle layer,
293 *stratum compactum* (sc) which consists of dense irregular connective tissue; and the *stratum*
294 *spongiosum* (ss) which is comprised of irregular loose connective tissue containing numerous
295 small smooth muscle bundles (Figure 4b, c). These smooth muscle bundles lie evenly along
296 the ventral aspect of the pouch. The anal fin is located caudal to the anal opening and cranial
297 to the brood pouch opening (Figure 4b). As in females, the skeletal muscles attaching to the
298 anal fin are organised into bilateral pairs, (Figure 4d). The anal fin skeletal musculature is
299 cranial to the pouch and caudal to the abdomen of the male seahorse, extending from the
300 ventral surface of the vertebral column to the inner ventral wall of the abdominal cavity,
301 where the muscle bundles attach to the anal fin pterygiophores (Figure 4d). The cartilaginous
302 pterygiophores are comprised of proximal radials, middle radials, and distal radials with anal
303 fin rays attaching to each distal radial (Figure 4b, d). The ASM is positioned within the
304 anterior portion of the pouch lumen as shown in transverse sections taken cranial to the pouch
305 opening (Figure 4e).
306



307
308 ***Figure 4.*** Whole body sections of a male seahorse, *Hippocampus abdominalis* excluding the
309 head and distal tail and stained with Cason's Trichrome. *(a)* A sagittal section of a male

310 seahorse. The brood pouch extends over the base of the tail (TSM: tail skeletal muscle) and
311 has a large lumen (L). The brood pouch is separated from the abdominal cavity (AC) by anal
312 fin skeletal muscle assembly (ASM). *(b)* High magnification image of the brood pouch wall
313 of the male seahorse caudal to the pouch opening. The inner epithelium (ie) is a single layer,
314 which forms the paternal portion of the placenta; the inner layer (IL) consists of loose
315 connective tissue; the middle layer, *stratum compactum* (sc) consists of dense irregular
316 connective tissue; the *stratum spongiosum* (ss) is comprised of loose connective tissue
317 containing small smooth muscle bundles (hollow black arrowheads); and the outer epithelium
318 faces external seawater. *(c)* High magnification of the anal fin (AF), brood pouch opening
319 (red arrowhead), and anal pore (black arrowhead). Anal fin rays (AF) are attached to distal
320 radials (#), which are in turn attached to the fused middle radials (MR) [47]. Four distal
321 radials can be seen (#) in this image, however only one fin ray is visible due to the orientation
322 of the tissue at sectioning. Small smooth muscle bundles (hollow black arrowheads) are
323 present within the loose connective tissue, forming the outer layer, *stratum spongiosum* (ss)
324 of the brood pouch. *(d)* Higher magnification image of the anal fin skeletal musculature
325 (ASM). The anal fin skeletal muscles are attached to the proximal radials (+) and the fused
326 middle radials (MR), which attach to the distal radials (#). (e) Transverse section of a male
327 seahorse at the anterior end of to the brood pouch (cranial to the pouch opening). All scale
328 bars represent 2000 μm. Abdominal cavity (AC), anal fin skeletal muscle (ASM), anal fin
329 (AF), connective tissue (CT), dermal plates (DP), dorsal fin skeletal muscle (DSM),
330 gastrointestinal tract (GIT), anal pore (black arrowhead), inner epithelium (ie), smooth
331 muscle (hollow black arrowhead), inner layer (IL), kidney (K), pouch lumen (L), liver (Liv),
332 stratum spongiosum (ss), stratum compactum (sc), brood pouch opening (red arrowhead), tail
333 skeletal muscle (TSM), proximal radial (+), distal radial (#).

334 **Discussion**

335 Isotocin produced no measurable contractions in seahorse brood pouch *in vitro*, which was
336 unexpected because nonapeptide hormones have a conserved role in producing involuntary
337 contractions of the smooth muscles of the reproductive tract in other vertebrate species [e.g.
338 27, 29, 31-34, 37, 49]. Since the administration of isotocin produces parturition-like
339 behaviours in non-pregnant male seahorses [22], nonapeptide hormones may act via a
340 different mechanism in male parturition, via neural pathways stimulating skeletal muscles.
341 We characterised the bone and muscle structures surrounding the brood pouch opening, and
342 while the brood pouch contains smooth muscle (Figure 4), as identified by Kawaguchi *et al.*

[4], the muscles are located in small, scattered bundles, even at the pouch opening where they are most abundant. This contrasts with the prolific, thick layers of smooth muscle present in the amniote uterus [reviewed in 50, 51]. The comparative lack of smooth muscle in the seahorse brood pouch explains why isotocin did not induce measurable contractions in isolated strips of pouch tissue. A similar explanation has been proposed for the inability of nonapeptide hormones to induce measurable contractions in teleost ovaries that contain very little smooth muscle [27]. This result raises the question of how parturition is produced in seahorses.

Nonapeptide hormones have complex physiological and behavioural roles beyond smooth muscle contraction at parturition, including mediating various reproductive behaviours in teleost fishes [reviewed in 52]. The prominent anal fin skeletal musculature associated with the pouch entrance in male seahorses leads us to postulate that control of skeletal muscle contraction may facilitate seahorse parturition. Mating is preceded by elaborate courtship, in which males dilate their pouch opening and force water into the pouch by bending forward and contracting their bodies to inflate the pouch, often while anchored to a support via the muscular tail [53-55]. Similarly, before and during parturition, males bend the trunk towards the tail, pressing and then relaxing [55]. This "pressing" behaviour can be accompanied by brief gaping of the pouch opening, and a series of jerks. During parturition, jerking and pressing continues, the pouch opening gets gradually bigger, and groups of neonates are ejected intermittently with each movement [Whittington pers. obs., 22, 55].

The basic structure of the anal fin assembly is similar across both sexes of *H. abdominalis* but is much larger in the males than females and differs in orientation (Figure 2). The anal fin moves during seahorse parturition [55], but has little or no function in swimming [56, 57]. We propose that the large skeletal muscle bundles located near the male brood pouch opening may play a role in deforming the pouch opening and expelling neonates at parturition, perhaps in concert with actions of the scattered smooth muscle bundles near the pouch entrance as suggested by Kawaguchi *et al*. [4]. Since fin inclinator muscles actuate fin ray assemblies in a side-to-side motion [47], it is possible that their simultaneous contraction along with flexible cartilaginous pterygiophores could deform the pouch opening. In concert with contraction of tail musculature facilitating the "pressing" behaviour, the effect would be to force water into the pouch and neonates out of the pouch with each thrust. Considering the observations of this study, and the effect of exogenous isotocin on males [22], we speculate

377 that seahorse parturition is facilitated by contractions of skeletal muscles, and that
378 nonapeptide hormones are involved in producing the cascade of behaviours leading to birth.
379 Future biomechanical and electrophysiological studies examining the contractility of the anal
380 fin assembly are required to test these hypotheses.

381 Acknowledgements

382 The authors thank Emma Sherratt for valuable advice on staining and imaging protocols. We
383 also acknowledge the facilities and the scientific and technical assistance of the Australian
384 Centre for Microscopy and Microanalysis and the Bosch Institute at the University of
385 Sydney. Alice Buddle, James Collison, Charles Foster, Jacqueline Herbert, Stephanie Liang,
386 Anna Roniotis, and Zoe Skalkos assisted with animal husbandry and/or laboratory work. This
387 work was supported by ARC DP180103370 to CMW and MBT and a Sydney Research
388 Accelerator (SOAR) fellowship to CMW.

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Supplementary Materials

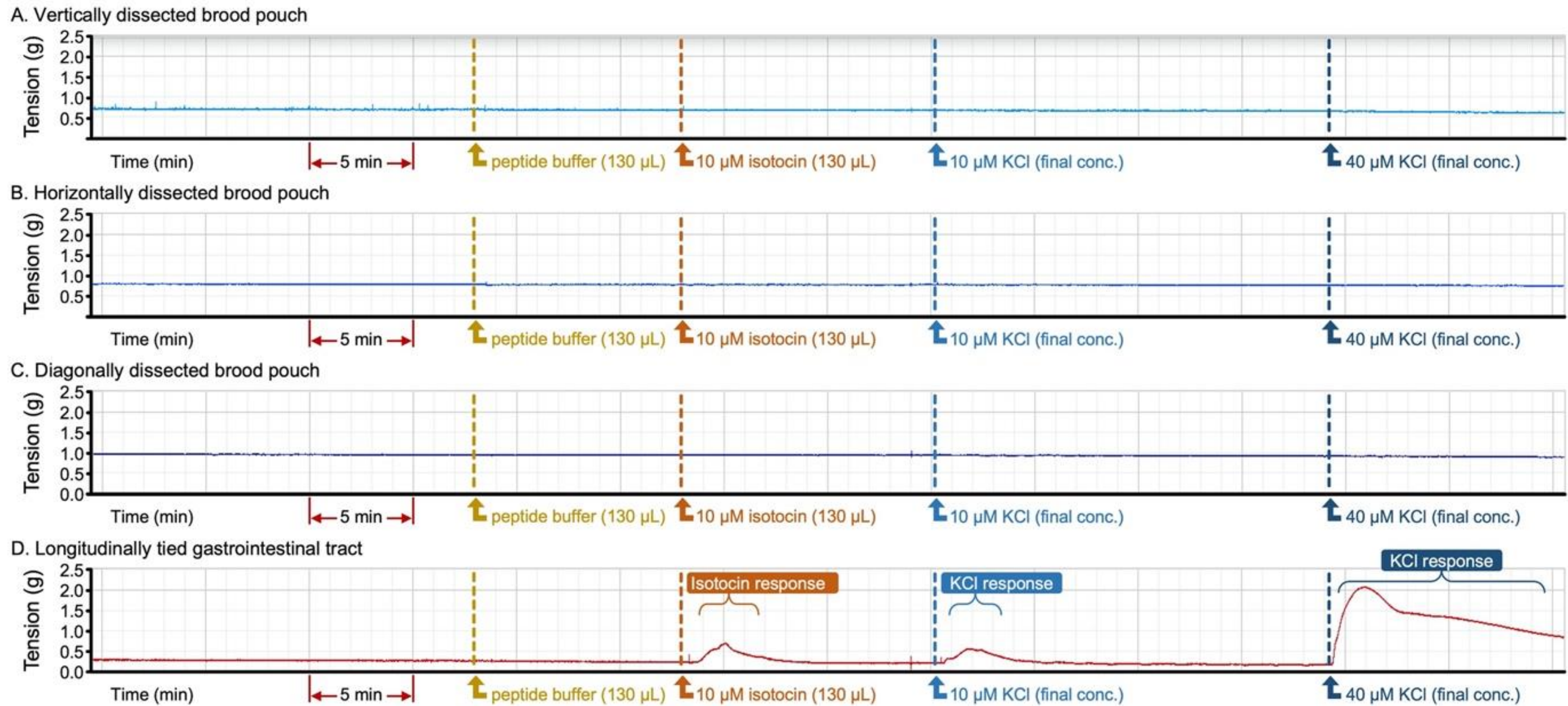

Figure S1. Representative partial contraction traces for individual strips of seahorse (*Hippocampus abdominalis*) brood pouch tissue (panels A - C) and gastrointestinal tract (positive control; panel D) suspended in marine Ringer's solution. These representative data are from a single late-stage pregnant male seahorse. Panels A - C are seahorse brood pouch tissue cut in vertical, horizontal, and diagonal orientation, respectively.

Panel D is longitudinally tied gastrointestinal tract. Brood pouch tissue (of any dissection orientation) did not contract in response to isotocin (10 μM) or KCl (10 and 40 μM), whereas gastrointestinal tract contracted in response to both isotocin and KCl. Consistent results were observed when tissues were suspended in Munsick's solution (Figure 1).

Video S1. Micro-CT scan video of an adult male seahorse (*Hippocampus abdominalis*). Reviewer link: https://drive.google.com/drive/folders/1qSAHsu1LdGPIRWyJ5TnJXBMTs8Dzs21H?usp=sharing

Video S2. Micro-CT scan video of an adult female seahorse (*Hippocampus abdominalis*). Reviewer link: https://drive.google.com/drive/folders/1qSAHsu1LdGPIRWyJ5TnJXBMTs8Dzs21H?usp=sharing